AD_____

Award Number: W81XWH-10-1-0189

TITLE: Heterobivalent Imaging Agents for Simultaneous Targeting Prostate-Specific Membrane Antigen (PSMA) and Hepsin

PRINCIPAL INVESTIGATOR: Dr. Youngjoo Byun

CONTRACTING ORGANIZATION: The Johns Hopkins University
Baltimore, MD 21216

REPORT DATE: April 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 01-04-2011 | Annual | 1 APR 2010 - 31 MAR 2011 |

**4. TITLE AND SUBTITLE**

Heterobivalent Imaging Agents for Simultaneous Targeting Prostate-Specific Membrane Antigen (PSMA) and Hepsin

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-10-1-0189

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Dr. Youngjoo Byun

E-Mail: ybyun1@jhmi.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

The Johns Hopkins University
Baltimore, MD 21216

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

We hypothized that the sensitivity and accuracy of prostate cancer diagnosis can be improved by dual-targeting of PSMA and hepsin. In order to provide imaging agents of enhanced affinity/avidity for prostate cancer, Two key components , the PSMA-binding ligand scaffold and the thiadiazole-derived hepsin-binding moieties, were prepared in the Year 1. The ultimate goals throughout the 3-yr project period which synthesize the heterobivalent conjugates of PSMA-ligand with three different types of hepsin ligands and evaluate their in vitro and in vivo biological properties using the optical- or nuclide-labeled conjugates will be accomplished by using the established synthetic methodologies in Year 1.

**15. SUBJECT TERMS**

PSMA, Hepsin, Prostate Cancer, Molecular Imaging

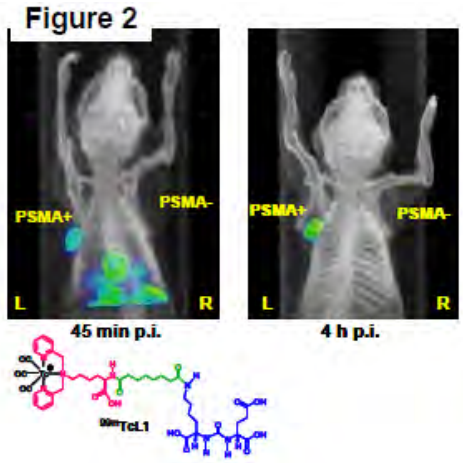
| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 9 | **19b. TELEPHONE NUMBER** *(include area code)* |

## A. Introduction

Prostate cancer (PCa) is the leading cancer in the U.S. population and the second leading cause of cancer death in men (1). Even if current detection methods of PCa using prostate-specific antigen (PSA) testing have advanced significantly for the diagnosis of patients with PCa, the controversy on PSA is currently still being debated. Due to the lack of PSA specificity for PCa, unnecessary biopsies or treatment of what would be benign or indolent disease. Therefore, there is an emerging need to detect small lesions, i.e., recurrent tumors in the surgical bed, local lymph node invasion and other subtle manifestations of the disease in men with an elevated serum PSA but no other obvious symptoms.

Prostate-specific membrane antigen (PSMA) is a type II integral membrane protein that has abundant and restricted expression on the surface of prostate carcinomas, particularly in androgen-independent, advanced and metastatic disease (2), (3). PSMA possesses the criteria of an ideal target for immunotherapy, i.e., expression primarily restricted to the prostate, abundantly expressed as protein at all stages of the disease, presented at the cell surface but not shed into the circulation, and association with enzymatic or signaling activity (3). Due to high sensitivity and non-invasive detection, imaging techniques such as magnetic resonance spectroscopy (MRS), positron emission tomography (PET) and single photon emission computed tomography (SPECT) as important medical and research tools to measure body functions are gaining favor over the anatomic techniques of computed tomography (CT) and MR, which merely detect enlarged tissue, revealing nothing of its underlying physiology. SPECT-CT scan of PCa using $^{111}$In-capromab pendetide (Cyt-356, ProstaScint), a [$^{111}$In]-labeled monoclonal antibody to prostate-specific membrane antigen (PSMA), showed promise in the clinic for identifying candidates for salvage radiotherapy. (4, 5)

Because of the important functions of PSMA for PCa, we sought initially to produce PSMA-imaging probes in order to measure its activity *in vivo* in a variety of conditions using PET and SPECT.(6) The S1' pocket is more selective than the S1, which is best bound with a glutamate residue, allowing us to take advantage of the structural freedom provided by the S1 pocket for introduction of imaging moieties. We chose the lysine in the S1 binding site as a core scaffold in order to 1) take advantage of the many radiohalogenation methods and radiohalogenated prosthetic groups developed previously for reaction with the ε-amino group of lysine residues, and 2) increase the structural diversity of urea-based PSMA inhibitors.(7, 8) Among several lysine analogues, 2-[3-[1-carboxy-5-(4-[$^{125}$I]iodo-benzoylamino)-pentyl]-ureido]-pentanedioic acid ([$^{125}$I]DCIBzL, 0.01 nM in Figure 1) and [$^{99m}$Tc]L1 (10 nM, in Figure 2) showed the best properties for PSMA imaging *in vivo*.(7, 8) A tunnel-like region linked to the S1 binding pocket projects toward the surface of the enzyme. Armed with this information and our own molecular modeling, we demonstrated that a linker of > 20Å occupying the tunnel region was needed between the S1 pocket and the bulky labeling groups including radiometal-chelators and optical-dyes.(7) [$^{99m}$Tc]**L1** has Lys-urea-Glu and bulky [$^{99m}$Tc(CO)$_3$]with a spacer length of > 20Å and demonstrated suitable inhibitory capacity *in vitro* as well as having provided clear



tumor delineation *in vivo*, with little background at 4 h post-injection (Figure 2).(7) More recently, Low and co-workers reported promising *in vivo* imaging results of PSMA-targeted [$^{99m}$Tc]-chelate complexes with > 20Å spacer containing Phe residues between bulky-chelating group and Glu-urea-Glu moiety.(9)

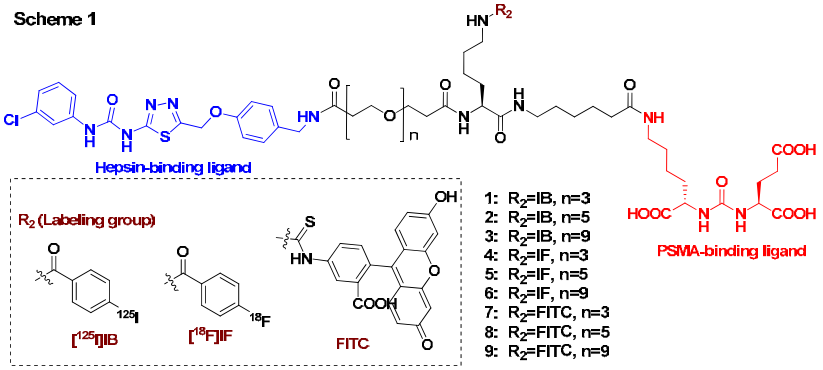
Hepsin also exhibited staining predominantly in the plasma membrane and was preferentially expressed in neoplastic prostate over benign prostate.(10) In addition, the mRNA level of *hepsin* was elevated in ~ 90% of PCa specimens and was > 10-fold higher in metastatic PCa than in normal prostate or benign prostatic hyperplasia (BPH). Hepsin is composed of 413 amino acids and a 373-residue is in extracellular region. The extracelluar 255 amino acids at the C-terminus, so called serine protease domain, are highly homologous among typeII trypsin-like serine proteases (TTSPs).(11) Recently, Chevillet et. al carried out high through-put screening of >10,000 molecules with purified human hepsin and identified 16 compounds with $IC_{50}$s of 0.23-2.31 μM and relative selectivity of up to 86-fold or greater compared to trypsin and thrombin.(12) However, no small-molecule hepsin inhibitors with high binding affinity ($K_i$ < 10 nM) has been reported to date even if hepsin is considered as one of key biomarkers in the progression and metastasis of PCa. This level ($K_i$ < 10 nM) is the initial cut-off for *in vivo* imaging studies.

Valency is the number of separate connections that one microscopic entity makes with another (13). Although originating in the idea of chemical bonding, *e.g.*, oxygen and nitrogen gases posses double and triple bonds, respectively, in biological terms we refer to the number of binding interactions between two species. In general, higher valency yields higher affinity. In the realm of nanoparticles, valency approach becomes even more important because they can accommodate more than just a few functional groups. Recently, Kelly et. al reported the successful detection of prostate cancer at the *in vivo* animal studies using hepsin-targeted multivalent nanoparticles.(14) Moderately-potent peptide (IPLVVPL, 120 nM) conjugated with fluorescent-labeled nanoparticle improved binding affinity/avidity for hepsin and exhibited fluorescent signal *via* FACs by >10-fold higher than the peptide alone. The ultimate goal, perhaps within reach of a molecular imaging agent – because such agents report on biology – would be to provide a compound that could predict which tumors would progress rapidly and which would not. We believe that our heterobivalent conjugates, which targets PCa-overexpressed PSMA and hepsin simultaneously, will possess the necessary characteristics to meet those challenges. The heterobivalent agents proposed here will be easily extendable to heteromultivalent agents by conjugating them to nanoparticles, but they are beyond the scope of the current proposal. From a standpoint view of synthesis, we intend to increase the chemical space that binds to PSMA/hepsin, in particular, the hepsin-binding region because the current known small molecule inhibitors possess only weak to moderate affinity.

## B. Specific Aims

We hypothized that the sensitivity and accuracy of PCa diagnosis can be improved by dual-targeting of PSMA and hepsin. We proposed heterobivalent conjugates of PSMA/hepsin-binding ligands labeled with optical dyes, positron- and gamma-emitting nuclides, in order to provide agents of enhanced affinity/avidity for PCa. The PSMA-binding ligand moiety of proposed conjugates is Lys-urea-Glu, which has been used as

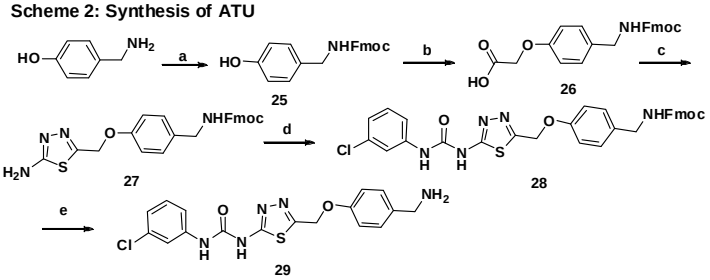
Scheme 1




the key S1'-probing moiety for preparation of our PSMA imaging probes. We proposed three hepsin-binding ligands based on the small molecules reported as weak to moderate hepsin inhibitors. These are 1) thiadiazole-containing scaffold identified through by high through-put screening of >10,000 compounds,(12) 2) indole-5-carboximidamide scaffold crystallized with hepsin,(15) and 3) IPLLVVPL peptide obtained by phage-display microarrays.(14) The ultimate goals throughout the 3-yr project period was to synthesize the heterobivalent conjugates of PSMA-ligand with three different types of hepsin ligands and to evaluate their in vitro and in vivo biological properties using the optical- or nuclide-labeled conjugates. In the 1st Year, we proposed the synthesis and evaluation of heterobivalent conjugates of PSMA-ligand and thiadiazole-derived hepsin ligand as shown in Scheme 1. The proposed hetero-bivalent conjugates shown in Scheme 1 consist of three moieties that possess distinct roles for dual targeting of PSMA and hepsin: (1) a high-affinity urea-based PSMA ligand, Lys-NHC(=O)NH-Glu which our group have developed, (2) a low M.W. thiadiazole-derived hepsin ligand that have moderate binding affinity ($IC_{50}$ = 0.5-5 μM) for hepsin, and (3) an easily-modified scaffold for introducing radionuclides, i.e., $^{125}I$ or $^{18}F$, or optical dyes. In the original proposal, we planned to prepare 9 conjugates (Scheme 1) in non-radiolabeled form in the Year 1.

## C. Accomplishments in Year 1

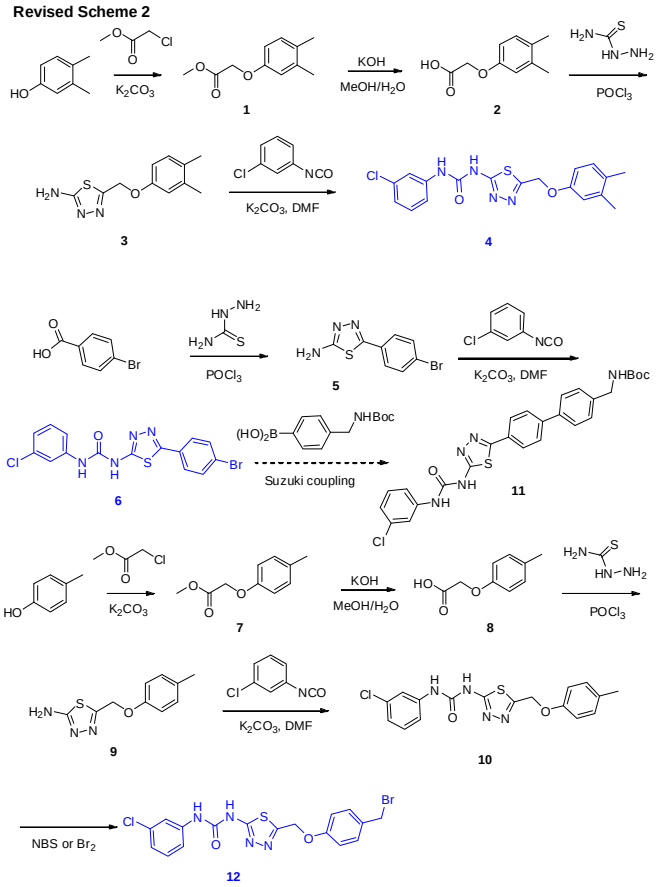
Scheme 2: Synthesis of ATU



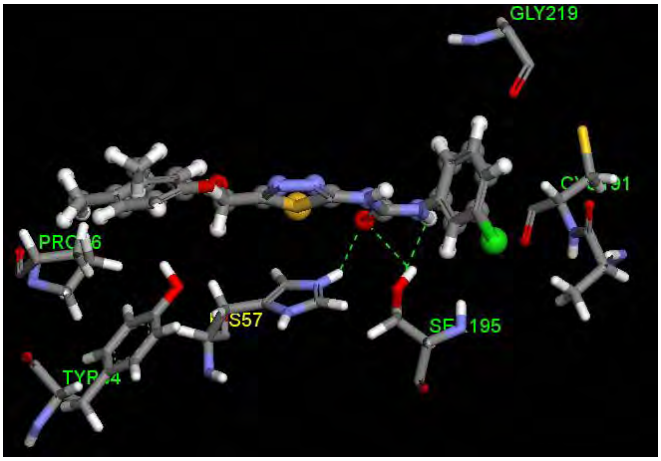

Reagents: (a) Fmoc-OSu, (b) i: ethylchloroacetate, $K_2CO_3$, DMF, ii: KOH, (c) i; hydrazinecarbothiomaide, HATU, $NEt_3$, DMF, ii: $PBr_3$, (d) 3-chlorobenzoisocyanate, (e) Piperidine/DMF

The original synthetic route to hepsin-binding ligand 1-(5-((4-(aminomethyl)phenoxy)methyl)-1,3,4-thiadiazol-2-yl)-3-(3-chlorophenyl)urea (ATU) is outlined in Scheme 2. However, Fmoc group of compound **25** was removed under the basic condition ($K_2CO_3$, DMF), which interfered with the ring formation of thiadiazole ring at the preparation step of compound **27**. Therefore, we made appropriate changes of the synthetic scheme to prepare the hepsin-binding

scaffolds. The revised synthetic route for ATU analogs is outlined in the revised Scheme 2. By applying the revised procedure, we prepared three hepsin-binding ligands (compounds **4, 6** and **10** in revised Scheme 2).



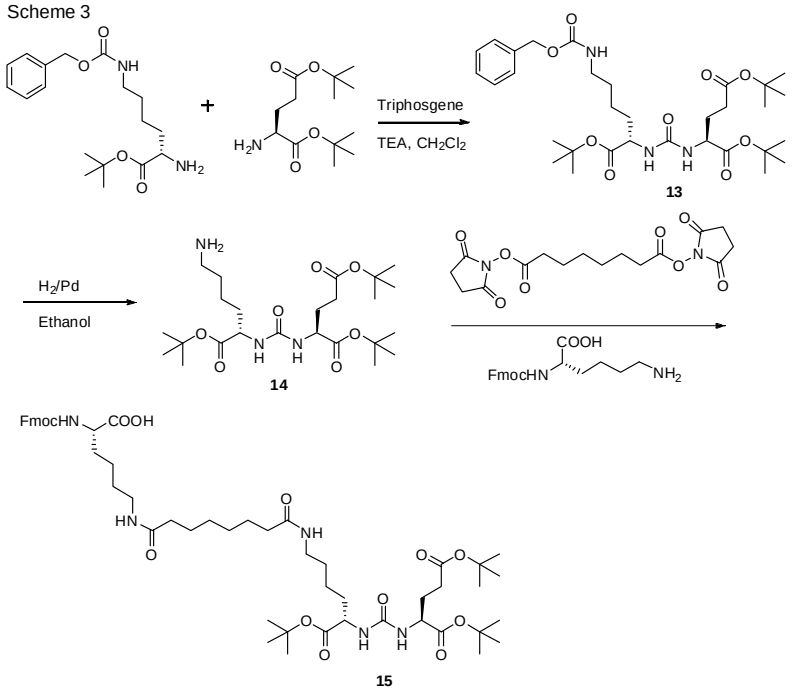

Briefly, 3,4-dimethylphenol reacted with methyl chloroacetate in DMF in the presence of potassium carbonate at room temperature to give the compound **1** in 75% yield. Methylester group of **1** was removed by the treatment of potassium hydroxide in water/methanol/tetrahydrofuran (2/2/1) to afford the compound **2** in 68% yield. Reaction of **2** with hydrazinecarbothioamide in phosphorous chloride under reflux provided the thiadiazole analog **3** in 58% yield. At this step, the reaction mixture should be slowly added to the iced water to prevent the explosive reaction of the remaining phosphorus chloride with water (*Caution*). Compound **3** reacted with 3-chlorophenylisocyanate in the presence of $K_2CO_3$ in DMF at 125 $^oC$ for 24 hrs to afford the urea analog **4** in 55% yield, which was reported as a hepsin inhibitor previously. (12) Compound **10** was also prepared in a similar way to the synthesis of compound **4** by using 4-methylphenol instead of 3,4-dimethylphenol as a starting material. Treatment of **10** with NBS underwent the radical bromination at the benzylic position to give compound **12**. The brominated compound **12** will react with the incoming nucleophilic groups such as alcohol or amines of PEG linkers. The synthesis of compound **6** was also outlined in the revised Scheme 2. Reaction of **6** with Boc-protected aminomethyl phenylboronic acid through the palladium-catalyzed Suzuki coupling will afford compound **11**. Treatment of **11** with trifluoroacetic acid (TFA) will give the tBoc-removed compound which can be easily conjugated with the PEG linker group.




In vitro $IC_{50}$ of compound **4** with hepsin protein was in the similar range of 0.76 μM.(12) Docking studies of **4** (left figure) in hepsin crystal structure using CDOCKER program (DS 3.0, Accelrys Inc.) also showed the plausible binding mode of compound **4** in the active site of hepsin, showing that urea carbonyl group of **4** is located close to the catalytic site and make hydrogen-bonding with His 57 (2.26 Å) and Ser 195 (2.63 Å).

The other key component, PSMA-binding scaffold (compound **15**), was prepared by following the synthetic methodology which our group have developed.

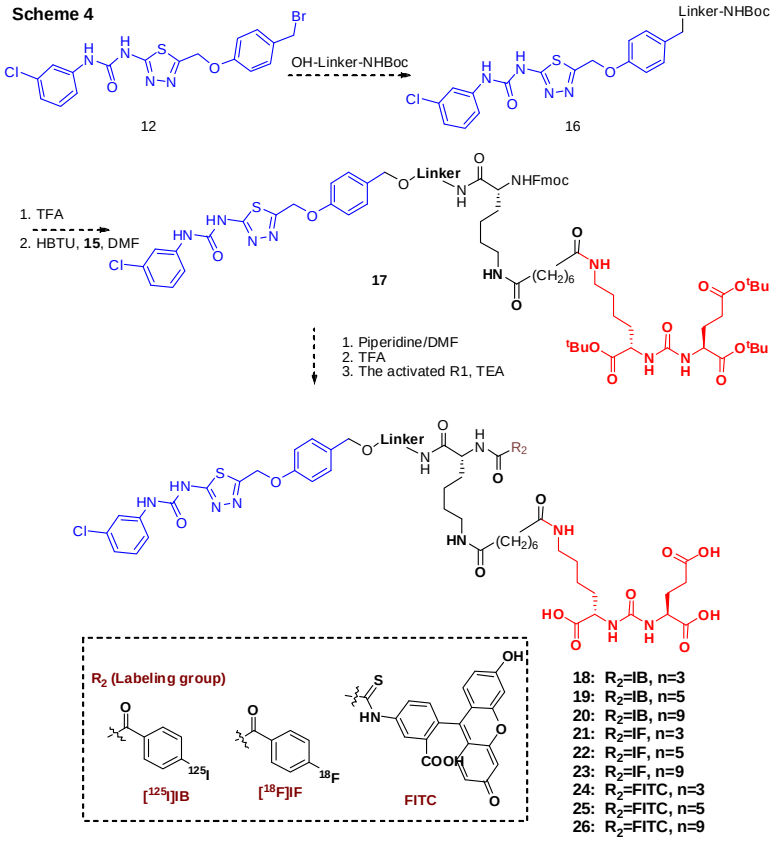
Scheme 3




Briefly, the protected Lys-urea-Glu **13** was synthesized by reacting the commercially available tBoc-protected glutamic acid with triphosgene in TEA/$CH_2Cl_2$ solution, followed by the addition of the protected lysine at -78 $^{o}$C. Hydrogenation reaction ($H_2$/Pd in ethanol) of **13** removed benzyl group to afford the Lys-Urea-Glu **14** in 85% yield**.** Compound **14** was conjugated with the suberic acid bis-(*N*-hydroxysuccinimide (DSS) in DMF, followed by the addition of Fmoc-protected lysine, gave the compound **15** in 48% yield.

Conjugation of **15** with the hepsin-binding ligand is described in Scheme 4. The hepsin-ligand **12** will be linked to PEG spacer using $S_N2$-type replacement reaction to afford compound **16.** Removal of tBoc group of **16** and subsequent conjugation with compound **15** by using the traditional peptide coupling condition will give compound **17**. Deprotection of Fmoc group and t-Bu group will be achieved by treating 20% piperidine in DMF and TFA, respectively. Reaction of the deprotected analog **17** with N-succinimidyl-4-[$^{125}$I]iodobenzoate(S[$^{125}$I]IB) or N-succinimidyl-4-[$^{18}$F]fluorobenzoate(S[$^{18}$F]FB) in the presence of TEA will provide the corresponding products **18-23**, respectively. For the synthesis of nonradiolabeled compounds, nonradioactive SIB and SFB will be used instead of S[$^{125}$I]IB and S[$^{18}$F]FB at the final step. In the similar way, coupling of **17** with fluorescein isothiocyanate (FITC) will give compounds **24-26** for optical imaging studies.

Scheme 4




**18:** $R_2$=IB, n=3
**19:** $R_2$=IB, n=5
**20:** $R_2$=IB, n=9
**21:** $R_2$=IF, n=3
**22:** $R_2$=IF, n=5
**23:** $R_2$=IF, n=9
**24:** $R_2$=FITC, n=3
**25:** $R_2$=FITC, n=5
**26:** $R_2$=FITC, n=9

**D. Research Plan for the 2nd and 3rd Year – New Milestones.**

The 1st-year portion of the original grant was to synthesize nine PSMA-hepsin conjugates in the non-radiolabeled form and to carry out *in vivo* animal imaging studies using the radiolabled or optical probes. However, we have had a difficulty in synthesizing PSMA-hepsin conjugates due to the fact that the original approach was not successful to obtain the proposed compounds. We redesigned the synthetic route to prepare the proposed conjugates and made the significant progress as discussed in the Section C. We believe that continued funding for the next two years could get us to the point of viable, new agents fairly soon. Even if the principal investigator moved to the Korea University (KU) in South Korea from the Johns Hopkins University (JHU), the new milestones reflects this change and allocate efficiently the work-duties at KU and JHU. The new milestones are realistic and our quest for imaging agents of prostate cancer will be accomplished by the end of the grant funding period. With some changes but not all of the resources of the original budget, we believe we can obtain PSMA-hepsin imaging probe sufficient for *in vivo* animal studies in the Year 2.

**Milestone:** *Synthetic chemistry, radiochemistry and biological evaluation on proposed conjugates*.

We will have a major on-going effort to synthesize the nonradiolabeled conjugates of PSMA-hepsin by a postdoc at KU. Dr. Byun, the principal investigator of this project, gets 100% salary support from KU and does not need any salary budget for Years 2-3. In the original proposal, we allocated 25% FTE of Dr. Byun and 40% FTE of a postdoc (TBN), who was scheduled to be recruited in 2011. But, we did not make the sufficient money to support the remaining 60% salary of the TBN postdoc and could not hire a postdoc. However, with the affiliation change of the principal investigator, we can hire a postdoc within the original budget by allocating the salary of Dr. Byun to that of a postdoc. Dedication of 100% time efforts of a postdoc to the project will accelerate the progress of the project in Years 2-3. As of March 1st 2011, Dr. Byun took an assistant professorship at the College of Pharmacy at KU which provides him with a spacious laboratory and equipments to carry out the synthetic chemistry and *in vitro* biological studies (Please see the detailed information of the equipments in the below). During the remaining period (2011-2013), we will complete the synthesis of nonradiolabeled conjugates and the biological evaluation *in vitro* at KU and carry out the radiolabeling chemistry and *in vivo* animal imaging studies at JHU. Dr. Byun and a postdoc at KU will carry out synthetic chemistry and in vitro binding studies at KU. Drs. Pomper and Mease, collaborators of this project, will oversee the radiolabeling phase and *in vivo* animal imaging studies. During the summer and winter breaks, Dr. Byun or a post-doc will visit JHU to execute radiochemistry and *in vivo* experiments. Dr. Byun has an adjunct faculty position at JHU and can get an access to the JHU facilities and equipments

**Facilities & Other Resources at KU**

Dr. Byun's laboratory consists of 930 sq. ft. of wet chemistry space in the College of Pharmacy Building at KU, completed in 4/2011. The chemistry facilities are fully equipped for standard organic synthesis in addition to eight (8) fume hoods for chemical synthesis. He has access to resources of KU College of Pharmacy including 600 MHz NMR, 300 MHz NMR, Confocal microscope, Flow cytometer, Triple

Quadrupole LC-MS, Q-ToF LC/MS, Fluorescence microplate reader, Agilent HPLC System, Lyophilizer, and $CO_2$ incubator.

**Reference Cited:**


1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin 2003; 53: 5-26.
2. Schulke N, Varlamova OA, Donovan GP, et al. The homodimer of prostate-specific membrane antigen is a functional target for cancer therapy. Proc Natl Acad Sci U S A 2003; 100: 12590-5.
3. Huang X, Bennett M, Thorpe PE. Anti-tumor effects and lack of side effects in mice of an immunotoxin directed against human and mouse prostate-specific membrane antigen. Prostate 2004; 61: 1-11.
4. Chang SS, Heston WD. The clinical role of prostate-specific membrane antigen (PSMA). Urol Oncol 2002; 7: 7-12.
5. Koontz BF, Mouraviev V, Johnson JL, et al. Use of Local 111In-Capromab Pendetide Scan Results to Predict Outcome After Salvage Radiotherapy for Prostate Cancer. Int J Radiat Oncol, Biol, Phys 2008; 71: 358-61.
6. Pomper MG, Musachio JL, Zhang J, et al. 11C-MCG: synthesis, uptake selectivity, and primate PET of a probe for glutamate carboxypeptidase II (NAALADase). Mol Imaging 2002; 1: 96-101.
7. Banerjee SR, Foss CA, Castanares M, et al. Synthesis and Evaluation of Technetium-99m- and Rhenium-Labeled Inhibitors of the Prostate-Specific Membrane Antigen (PSMA). J Med Chem 2008; 51: 4504-17.
8. Chen Y, Foss CA, Byun Y, et al. Radiohalogenated Prostate-Specific Membrane Antigen (PSMA)-Based Ureas as Imaging Agents for Prostate Cancer. J Med Chem 2008; 51: 7933-43.
9. Kularatne SA, Zhou Z, Yang J, Post CB, Low PS. Design, Synthesis, and Preclinical Evaluation of Prostate-Specific Membrane Antigen Targeted 99mTc-Radioimaging Agents. Mol Pharmaceutics: ACS ASAP.
10. Dhanasekaran SM, Barrette TR, Ghosh D, et al. Delineation of prognostic biomarkers in prostate cancer. Nature (London, U K) 2001; 412: 822-6.
11. Somoza JR, Ho JD, Luong C, et al. The Structure of the Extracellular Region of Human Hepsin Reveals a Serine Protease Domain and a Novel Scavenger Receptor Cysteine-Rich (SRCR) Domain. Structure (Cambridge, MA, U S) 2003; 11: 1123-31.
12. Chevillet JR, Park GJ, Bedalov A, Simon JA, Vasioukhin VI. Identification and characterization of small-molecule inhibitors of hepsin. Mol Cancer Ther 2008; 7: 3343-51.
13. Tweedle MF. Adventures in multivalency, the Harry S. Fischer memorial lecture CMR 2005; Evian, France. Contrast Media Mol Imaging 2006; 1: 2-9.
14. Kelly KA, Setlur SR, Ross R, et al. Detection of Early Prostate Cancer Using a Hepsin-Targeted Imaging Agent. Cancer Res 2008; 68: 2286-91.
15. Katz BA, Luong C, Ho JD, et al. Dissecting and Designing Inhibitor Selectivity Determinants at the S1 Site Using an Artificial Ala190 Protease (Ala190 uPA). J Mol Biol 2004; 344: 527-47.